Identification of PtdIns(3,4)P_2 effectors in human platelets using quantitative proteomics

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Abstract

After decades in PtdIns(3,4,5)P_3's shadow, PtdIns(3,4)P_2 has now emerged as a bona fide regulator of important cellular events, including endocytosis and cell migration. New understanding of PtdIns(3,4)P_2's cellular roles has been possible via novel approaches to observe and quantify cellular PtdIns(3,4)P_2 dynamics, alongside methods to target the kinases and phosphatases governing phosphoinositide turnover. Despite this, the mechanisms by which PtdIns(3,4)P_2 orchestrates its cellular roles remain more poorly understood, most notably because, to date, few PtdIns(3,4)P_2 effectors have been identified. Here, we develop and apply an affinity-proteomics strategy to conduct a global screen for PtdIns(3,4)P_2 interactors in human platelets; a primary cell type with striking PtdIns(3,4)P_2 accumulation. Through an integrated approach, coupling affinity capture of PtdIns(3,4)P_2-binding proteins to both label-free and isobaric tag-based quantitative proteomics, we identify a diverse PtdIns(3,4)P_2 interactome. Included are long-established PtdIns(3,4)P_2-binding proteins such as PLEKHA1, PLEKHA2, AKT and DAPP1, and a host of potentially novel effectors, including MTMR5, PNKD, RASA3 and GAB3. The PtdIns(3,4)P_2 interactome shows an enrichment of pleckstrin homology (PH) domain-containing proteins, and through bioinformatics and array analyses we characterise the PH domain of MTMR5 and define its phosphoinositide selectivity. The interactome is also diverse in function, including several proteins known to support protein trafficking and cytoskeletal mobilisation. Such proteins have the ability to drive key platelet events, and to fulfil recently-defined roles for PtdIns(3,4)P_2 in a wider range of cell types. Moreover, this study will serve as a valuable resource for the future characterisation of effector-driven PtdIns(3,4)P_2 function.

1. Introduction

Phosphoinositides regulate diverse and essential cellular processes, most commonly via interactions with versatile sets of effector proteins. The best-characterised example is the Class I phosphoinositide 3-kinase (PI3K) product, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), which coordinates the plasma membrane recruitment and/or activity of effectors such as AKT, BTK and ARAP1 to control cellular processes including metabolism, growth and migration. In contrast to the extensive functional characterisation of PtdIns(3,4,5)P_3 over the past three decades, phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) has been described as a "neglected phosphoinositide" [1] and has been relatively overlooked, largely because it was long considered to be little more than a by-product of PtdIns(3,4,5)P_3 signal termination by 5-phosphatases [1, 2]. However, work in recent years has shed new light on the importance of PtdIns(3,4)P_2 as a signalling phospholipid in its own right. Indeed, a number of studies have revealed roles for PtdIns(3,4)P_2 in the regulation of endocytosis [3, 4], membrane morphology [5, 6], cell polarization [7] and migration [8], while also suggesting significance in cancer [9-12].

Despite this new functional appreciation, PtdIns(3,4)P_2 remains enigmatic in many regards. While a number of phosphoinositide kinases and phosphatases have been proposed to contribute to the synthesis (e.g. PI3KC2α/β/γ, SHIP1/2, INPP5B/E/J, SYNJ2) and degradation (e.g. INPP4A/B, PTEN) of PtdIns(3,4)P_2, the complete set and balance of enzymes regulating the turnover of this phosphoinositide *in vivo*, and the details of their dynamic regulation, remain unclear and appear to be highly context dependent [2-4, 11, 13]. Moreover, although generally considered to act via the membrane recruitment of effector proteins in a manner analogous to PtdIns(3,4,5)P_3, mechanistic understanding of how PtdIns(3,4)P_2 regulates cell function following synthesis remains limited, because only a modest number of effectors have been identified [14].

Of the known PtdIns(3,4)P_2 effectors, among the best-characterised are those also regulated by PtdIns(3,4,5)P_3; most notably AKT and PDK1. The functional significance of this dual affinity and the relative contribution of the two lipids to protein regulation in specific signalling contexts is challenging to dissect. Indeed it has been proposed that PtdIns(3,4)P_2 can both support and inhibit Class I PI3K/PtdIns(3,4,5)P_3 signalling via both distinct and overlapping effectors [1, 2, 11, 15-18]. A small number of proteins have been reported to show clear selectivity for PtdIns(3,4)P_2 over other phosphoinositides - most notably PLEHKA1 and 2 (commonly known as TAPP1 and 2) [19-21], but also Lamellipodin [22] - and the characterisation of such effectors has helped uncover functional roles for PtdIns(3,4)P_2 in different cell types [16, 17, 23]. Despite this progress, the network of known PtdIns(3,4)P_2 effectors remains limited [14], and it is unclear whether PtdIns(3,4)P_2 is able to regulate a wider family of proteins comparable to that of PtdIns(3,4,5)P_3.

Global cellular PtdIns(3,4)P_2 accumulation in response to cell surface receptor activation is commonly slower and more sustained than that of PtdIns(3,4,5)P_3, although distinct phosphoinositide pools may display unique kinetics [2, 13, 24, 25]. Stimulated platelets generate a striking quantity of PtdIns(3,4)P_2 relative to many cell types [2, 11, 26-30] and, as their function is critically dependent on PI3K signalling [14], they represent an excellent primary cell model for the characterisation of 3-phosphoinositide biology. Furthermore, the use of platelets has previously supported advances in the understanding of PtdIns(3,4)P_2 behaviour and function [11, 26-28, 31], and thus these cells would serve as an excellent vehicle for the identification of PtdIns(3,4)P_2 effectors. While the precise functional role of PtdIns(3,4)P_2 remains unclear, its production in platelets is strongly dependent on $\alpha_{IIb}\beta_3$ integrin engagement, and has been proposed to support platelet aggregation and cytoskeletal arrangements required for thrombus formation [14, 26, 27, 29]. Its accumulation correlates with a decline of the initial rapid PtdIns(3,4,5)P_3 generation following cell surface receptor activation, and accordingly has been shown to be heavily dependent on the PtdIns(3,4,5)P_3 5-phosphatase, SHIP1 [32]. Platelets lacking SHIP1 show striking defects in platelet signalling and thrombus formation, potentially due to observed changes in the PtdIns(3,4,5)P_3/ PtdIns(3,4)P_2 balance [32, 33]. Here, we couple affinity capture of PtdIns(3,4)P_2-binding proteins from human platelet lysates, to label-free and isobaric tag-based quantitative mass spectrometry, in order to define a global cellular PtdIns(3,4)P_2 interactome. We present this dataset to support new understanding of PtdIns(3,4)P_2's role in platelets, but also as a general resource to facilitate improved understanding of PtdIns(3,4)P_2 effectors and their role in the regulation of important aspects of cell function.

2. Materials and methods

2.1 Materials

Antibodies against RASA3 (sc-166442 and sc-34468), PLEKHA1 (sc-50468), ADAP1 (sc-47836) and GAPDH (sc-25778) were from Santa Cruz Biotechnology (Insight Scientific, Middlesex, UK). The antibody against PHLDB1 (HPA038448) was from Sigma Aldrich (Poole, UK). The antibody against DAPP1 (AF7024) was from R&D Systems (Abingdon, UK). The antibody against GST (2622) and DyLight 680/800 secondary antibodies were from Cell Signaling Technologies (New

England Biolabs, Hitchin, UK). Anti-rabbit HRP-coupled secondary antibody was from Jackson ImmunoResearch (Stratech Scientific Ltd, Ely, UK). Immobilon FL polyvinylidene difluoride (PVDF) was from Merck Millipore (Dorset, UK). Odyssey blocking buffer (TBS) was from LI-COR Biosciences (Cambridge, UK). Blank control beads, PtdIns(3,4)P_2 beads (P-B034A), free PtdIns(3,4)P_2 (P-3408) and PIP Arrays (P-6100) were from Echelon Biosciences, distributed via Tebu-Bio (Peterborough, UK). cOmplete mini protease inhibitor tablets were from Roche Life Sciences (Welwyn Garden City, UK). Tandem Mass Tag reagents and NuPAGE sample buffer were from Thermo Fisher Scientific (Loughborough, UK). Leukocyte removal filters were from Pall (Portsmouth, UK). All other reagents were from Sigma Aldrich (Poole, UK) unless otherwise stated in the relevant methods subsection.

2.2 Human platelet preparation

Venous blood, drawn into a syringe containing 4% trisodium citrate (9:1, volume-to-volume), was obtained from healthy, medication-free, human volunteers. The study was conducted with the approval of the local research ethics committee at the University of Bristol, and volunteers gave full informed consent in accordance with the Declaration of Helsinki. Platelets were isolated as previously described [34], utilising a protocol developed to minimize plasma, erythrocyte, and leukocyte protein contamination. The platelet suspension was allowed to rest for 30 minutes at 30°C before use.

2.3 Affinity capture of platelet PtdIns(3,4)P_2-binding proteins

Purified platelets were centrifuged (520*g*, 10 minutes, room temperature), and the pellet was lysed in ice-cold lysis buffer (20 mM HEPES [pH 7.4], 120 mM NaCl, 0.5% NP40, 5 mM EGTA, 5 mM EDTA, 5 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, cOmplete mini protease inhibitor tablet). Phosphatase inhibitors were included to preserve the identity of PtdIns(3,4)P_2 on the beads [31, 35]. Lysates were freeze-thawed, vortexed, and centrifuged (12000*g*, 10 minutes, 4°C) to provide final clarified samples. A total of $8x10^8$ platelets were used per sample for proteomics experiments. Blank control beads or PtdIns(3,4)P_2-coupled beads were prewashed 3x with ice-cold lysis buffer by low speed centrifugation, before affinity capture was performed by incubating platelet lysate with the control or PtdIns(3,4)P_2-coupled beads for 90 minutes at 4°C under gentle rotation. For competition control samples, lysates were preincubated with 40 µM free PtdIns(3,4)P_2 for 30 minutes prior to PtdIns(3,4)P_2 bead incubation. Following the incubation, beads were washed 3x with either ice-cold lysis buffer, or ice-cold 20 mM HEPES [pH 7.4], 120 mM NaCl, 0.5% NP40 (for TMT samples), by low speed centrifugation. Proteins were either eluted from the beads by heating to 95°C in NuPAGE LDS sample buffer (plus 50 mM dithiothreitol) for 10 minutes (immunoblotting or label-free LC-MS/MS analysis), or trypsin-digested directly on the beads (TMT analysis), as discussed in the relevant Supplementary Methods sections.

2.4 Platelet PtdIns(3,4)P_2 interactome analysis by mass spectrometry

A brief description is given here, with full details in Supplementary Methods. For label-free experiments, pull down eluates (3 per donor, 3 independent donors) in sample buffer were run into an SDS-PAGE gel and each gel lane (eluate) cut into 5 slices. Each slice was subjected to in-gel trypsin digestion using a DigestPro automated digestion unit (Intavis Ltd, Koln, Germany). The resulting peptides were fractionated using an Ultimate 3000 nanoHPLC system in line with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Loughborough, UK). For TMT experiments, on-bead trypsin digestion was carried out for each pull down sample (3 per donor, 2 independent donors), peptides were labelled with Tandem Mass Tag (TMT) sixplex reagents according to the manufacturer's protocol (Thermo Fisher Scientific, Loughborough, UK), and the labelled samples were pooled. The pooled sample was evaporated to dryness, resuspended in formic acid and then desalted using a SepPak cartridge according to the manufacturer's instructions (Waters, Elstree, UK). Eluate from the SepPak cartridge was again evaporated to dryness and resuspended prior to fractionation by high pH reversed-phase chromatography using an Ultimate 3000 liquid chromatography system (Thermo Fisher Scientific, Loughborough, UK). High pH RP fractions were further fractionated using an Ultimate 3000 nano-LC system in line with an Orbitrap Fusion Tribrid

mass spectrometer (Thermo Fisher Scientific, Loughborough, UK). Full details of data analysis are given in Supplementary Methods.

2.5 Bioinformatics

Protein domain enrichment analysis was conducted using the InterPro output from DAVID [36, 37] version 6.8. Interaction network analysis was conducted using STRING [38] version 11, searching for experimentally-determined and databased-curated interactions, with a confidence score of 0.9. PH domain sequence alignment was carried out using Clustal Omega 1.2.4 [39]. The MTMR5 PH domain structure was rendered in PyMOL 2.1.1 using conformer 1 of PDB entry 1V5U, with the relevant amino acid side chains highlighted, as discussed in the text.

2.6 Immunoblotting

Pull down eluates and input samples were resolved by electrophoresis and transferred onto Immobilon FL PVDF membrane following standard procedures [40]. For some experiments, membranes were cut into sections to allow multiple proteins to be probed separately with different primary antibodies. Proteins were visualized by near-infrared detection using a LI-COR® Odyssey imaging system and DyLight 680/800 secondary antibodies. LI-COR® Image Studio was used to prepare figures.

2.7 MTMR5 PH domain expression

DNA sequence corresponding to residues 1752-1868 of human MTMR5 was optimised for *E. Coli* expression and cloned into the BamHI-EcoRI sites of pGEX-4-T1 (Genscript, New Jersey, USA). Protein was expressed in BL21 cells, purified from lysates using glutathione sepharose and eluted with 15mM reduced L-glutathione, as previously described [41].

2.8 MTMR5 phosphoinositide arrays

PIP array membranes were incubated in blocking buffer (3% fatty acid-free BSA in 20mM Tris, 150mM NaCl, 0.1% Tween (TBS-T)) for 1 hour at room temperature. Purified GST-MTMR5 PH domain was diluted in blocking buffer to a final concentration of 0.5µg/ml and incubated with the membrane for 1 hour at room temperature, before washing four times in TBS-T for 10 minutes per wash. The membrane was then incubated with anti-GST primary antibody at a dilution of 1:1000 in blocking buffer for 1 hour at room temperature, before a further four washes, and incubation with an HRP-conjugated anti-rabbit secondary antibody at a dilution of 1:10000 in blocking buffer for 1 hour at room temperature. After a final four washes of the membrane, the interaction between the MTMR5 PH domain and phosphoinositides was detected by enhanced chemiluminescence.

3. Results

We have previously utilised affinity proteomics to define the PtdIns(3,4,5)P_3 interactome of human platelets, allowing the identification and subsequent functional characterisation of Class I PI3K effectors regulating important platelet signalling events [34, 41]. We therefore set out to apply a similar approach to identify PtdIns(3,4)P_2 effectors, with the aim of providing a valuable data resource to advance understanding of this more poorly-characterised phosphoinositide. Affinity proteomics experiments were conducted using human platelet lysates as outlined in Figure 1. As platelet proteomics can be confounded by contamination of samples with proteins derived from blood plasma, or from co-purified leukocytes and erythrocytes, we utilised an optimised human platelet purification protocol [34] to obtain platelet fractions, and used freshly-isolated platelets to avoid proteome degradation. Furthermore, because affinity proteomics is inevitably prone to the capture of non-specific proteins, in addition to the use of PtdIns(3,4)P_2-coupled beads, we incorporated independent control samples utilising blank bead capture, or free-PtdIns(3,4)P_2 competition, to

confirm binding specificity. We also utilised fractionation at the protein and peptide level to reduce sample complexity and to limit crowding out of lower abundance interactors.

An initial global analysis of the label-free data across the three blood donors identified a number of known PtdIns(3,4)P_2-binding proteins in our screen, including the archetypal PtdIns(3,4)P_2 effectors, PLEKHA1, PLEKHA2 and DAPP1, thus validating our approach (Figure 2). Of note, visualising this analysis revealed the striking number of proteins demonstrating enrichment on the PtdIns(3,4)P_2 beads when compared to blank control beads (Figure 2A), and emphasized the importance of our dual controlled approach, with our free-PtdIns(3,4)P_2 competition experiments confirming only a proportion of the PtdIns(3,4)P_2 bead proteome to show PtdIns(3,4)P_2 specificity (Figure 2B). This highlights the challenge of identifying hits when relying on a single bait capture for identifying phosphoinositide-binding proteins (see Figure 2C for a range of protein behaviours), as has been the approach in several previous published screens, and the importance of a quantitative methodology. With this in mind, to obtain our final comprehensive PtdIns(3,4)P_2 interactome, we utilised a quantitative filtering approach within and across the label-free datasets of the three blood donors, followed by cross referencing with an independent dataset from multiplexed affinity proteomics experiments carried out utilising isobaric labelling of peptides with tandem mass tags (TMT). The latter approach permits pooling of samples at the peptide level, minimising technical variation in the LC-MS runs and providing a direct, ratiometric readout.

This integrated approach defined a diverse PtdIns(3,4)P_2 interactome (Table 1 & Table S1), including the aforementioned classically-described PtdIns(3,4)P_2-binding proteins, and additional endogenous platelet proteins for which PtdIns(3,4)P_2 affinity has been previously proposed via other approaches, such as ADAP1, PHLDB1 and SWAP70 [20, 21, 42-44]. Moreover, our dataset includes a range of further proteins likely to represent novel PtdIns(3,4)P_2-regulated proteins, including PNKD, RASA3, MTMR5, GAB3 and PLEKHO2. Importantly, while proteins such as DAPP1, AKT and RASA3 have been previously defined as important signalling nodes in human and mouse platelets [34, 41, 45, 46], the function of a large proportion of the proteins identified, including the well-established PtdIns(3,4)P_2 effectors PLEKHA1 and 2, remains unknown in this cell type, and so for many proteins this study is the first insight into their functional regulation. The specificity of our approach was validated by the absence of proteins specific for other phosphoinositides in our dataset; most notably PtdIns(3,4,5)P_3-specific effectors such as BTK, Tec, and DOCK-family proteins. Such proteins are highly expressed in platelets, and were abundantly purified in our previous PtdIns(3,4,5)P_3 interactome screen [34]. Furthermore, the absence of the known PtdIns(3,4)P_2-binding protein Lamellipodin [23], which is highly expressed in lymphocytes, but not in platelets [47], supports the purity of our platelet preparation.

We confirmed a subset of hits from our proteomics screen by immunoblotting (Figure 3), which gave further insights into the relative affinity of the proteins for PtdIns(3,4)P_2 under our experimental conditions. To our knowledge, this included the first evidence of the affinity of some of these full length, endogenous proteins for PtdIns(3,4)P_2 including, of note, RASA3, a protein critical for integrin signalling and platelet function [41, 48]. The DAPP1 PH domain has been reported to have high affinity for PtdIns(3,4)P_2 and, in agreement with previous work [34, 49], DAPP1 was captured highly efficiently on the PtdIns(3,4)P_2-coupled beads. PLEKHA1 was challenging to detect in platelet lysates by immunoblotting, suggesting it may be expressed at relatively low levels in this cell type, consistent with published expression data [47, 50]. Despite this, this protein was highly enriched on the PtdIns(3,4)P_2 beads, supporting the high affinity of the PLEKHA1/2 proteins for this phospholipid and suggesting they could likely respond adequately to localised PtdIns(3,4)P_2 in platelets.

Our interactome contained multiple PH domain-containing proteins (Figure 4A), which ontology analysis confirmed to be significantly enriched in the dataset (Figure 4B) in line with the recognized

importance of a subset of this protein domain family in PtdIns(3,4)P_2 (as well as PtdIns(3,4,5)P_3) binding [51]. This analysis also revealed MTMR1 and FRMD4B to possess pleckstrin homology-like domains which, in addition to cytohesin binding, may also support the regulation of the latter by PtdIns(3,4,5)P_3 [34]. Other protein domains associated with PtdIns(3,4,5)P_3 binding, such as the DHR-1 domain of DOCK family proteins [34, 52], were not enriched in the interactome, further supporting the specificity of our approach. Previous work has defined key residues within certain PH domains which are important for their interaction with PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 [51, 53-56]. We performed sequence alignment analysis of the PH domains identified in our screen, which confirmed several, including those of GAB3, MTMR5 and PLEKHO2, to possess a pattern of conserved basic residues in a previously-described KX_n(K/R)XR motif in the β1-β2 strand region [51, 54, 57] (Figure 4A). Interestingly, SWAP70 and OSBL8 diverged from this motif, with the latter lacking it completely, despite our study being in agreement with others that the PH domains of both of these proteins interact directly with PtdIns(3,4)P_2 [44, 58]. This can be explained by the presence of additional basic residues, including the RR motif in the β1-β2 loop of SWAP70 [44], and K/R residues in the β1-β2 and β5-β6 loops of OSBL8 [58], with PtdIns(3,4)P_2 likely binding the latter via a non-canonical basic pocket comparable to that of ARHGAP9 [56]. Further residues within PH domains also contribute to PtdIns(3,4)P_2- and PtdIns(3,4,5)P_3-binding [51, 57], while other regions of the protein may be important [59], making phosphoinositide effector identification challenging from a purely computational approach, and supporting the continued need for experimental identification.

Assessment of the structure of the PH domain of mouse MTMR5 confirmed a characteristic seven-stranded β-sandwich with a C-terminal α-helix (Figure 5A). Mapping of the highly conserved basic residues from our alignment of the MTMR5 PH domain onto this structure revealed their localisation to form a potential phosphoinositide-binding pocket comparable to that previously defined for DAPP1 and other PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 effectors [57, 60]. In this pocket, the basic amino acid sidechains can form multiple hydrogen bonds with the phosphates of the inositol headgroup [60], and thus these observations further support our identification of MTMR5 as a PtdIns(3,4)P_2 interactor. Comparison of the PH domain of MTMR5 to dual PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 effectors such as DAPP1 also suggested MTMR5 may have the potential to interact not only with PtdIns(3,4)P_2, but also with PtdIns(3,4,5)P_3. To assess this, and to also determine MTMR5's wider phosphoinositide selectivity, we expressed the PH domain of human MTMR5 as a GST-fusion protein (Figure 5B), and carried out lipid binding arrays against all phosphoinositides. This confirmed the affinity of the MTMR5 PH domain for PtdIns(3,4)P_2, while indeed also revealing some affinity for PtdIns(3,4,5)P_3 (Figure 5C).

A high confidence network analysis of our dataset using STRING [38] revealed minimal known protein-protein interactions amongst the proteins identified (Figure 6), suggesting the majority were captured directly on the PtdIns(3,4)P_2 beads, rather than being present as indirect components of the wider PtdIns(3,4)P_2 signalosome. Functional consideration of the platelet PtdIns(3,4)P_2 interactome reveals a number of signalling 'adaptors' lacking conventional catalytic function (e.g. DAPP1, PLEKHA1, PLEKHA2, GAB3, PHLDB1), in addition to enzymes (e.g. AKT, PDK1, PFKP, AGK) and regulators of GTPase activity (e.g. RASA3, MTMR5, ADAP1). The interactome also includes candidates associated with small molecule transport (e.g. SLC25A1, ORNT1), mitochondrial function (e.g. SQOR, MICU1, DECR1), and receptors (e.g. GPVI, P2RX1, F2RL3), highlighting the diversity of processes regulated by PtdIns(3,4)P_2, and opening avenues for novel exploration with regards to the cellular roles of this phospholipid.

4. Discussion

After years on the side-lines, a number of recent studies have shed light on the role of PtdIns(3,4)P_2 as a bona fide regulator of important cellular processes [3-5, 7-11]. The development of new tools and methodologies to manipulate enzymes in the PtdIns(3,4)P_2 synthetic pathway, and to observe and quantify PtdIns(3,4)P_2 (and other phosphoinositides) in biological contexts, has permitted new understanding of its synthesis and degradation, distinct cellular roles, and likely significance in disease [11, 13, 18, 61]. Despite these recent advances, knowledge of the effectors allowing PtdIns(3,4)P_2 to drive important cellular processes remains limited [14]. Furthermore, despite progress in other cell types, the mechanistic and functional action of this phospholipid in platelets remains poorly understood, despite observations of PtdIns(3,4)P_2 accumulation in platelets occurring three decades ago [26-28]. Indeed, the study of PtdIns(3,4)P_2 in platelets has remained largely limited to observations of the temporal patterns of synthesis and degradation, and potential roles in AKT regulation [11, 27, 29, 30, 62, 63]. PtdIns(3,4)P_2 accumulation in platelets is most striking following integrin $\alpha_{IIb}\beta_3$ engagement and platelet aggregation, and has been proposed to sustain or strengthen platelet aggregation, potentially in part by supporting cytoskeletal changes [14, 26, 29]. This relatively late accumulation of PtdIns(3,4)P_2 correlates with a decline in an initial, rapid PtdIns(3,4,5)P_3 spike following platelet activation, and is heavily dependent on SHIP1 5-phosphatase activity [32, 64, 65]. Class II PI3Ks can also produce distinct pools of PtdIns(3,4)P_2 to support events such as clathrin-dependent endocytosis at the plasma membrane and endosomal mTOR suppression in some cell types [3, 4]. However, while PI3KC2α plays an important role in platelet membrane structure and function, Class II PI3Ks do not appear to be clearly important for agonist-induced PtdIns(3,4)P_2 production in platelets [66-68].

Our proteomics screen confirmed that human platelets possess a core set of PtdIns(3,4)P_2 effectors observed previously using *in vitro* approaches or other cell types, including AKT, PDK1, DAPP1 and PLEKHA1 and 2. The AKT isoforms and PDK1 have important roles in platelet activation and thrombus formation in response to the activation of various cell surface receptors [45, 46], and we have previously defined a key role for DAPP1 downstream of glycoprotein VI [34]. In contrast, the roles of the tandem PH domain-containing adaptors PLEKHA1 and PLEKHA2 in platelets remain unknown. These adaptor proteins can provide negative feedback of insulin and PI3K signalling pathways, and in B cells PLEKHA1/2 can regulate cellular adhesion and migration, germinal center responses, and metabolism [1, 16, 17, 19, 69, 70]. The importance of the PtdIns(3,4)P_2-PLEKHA1/2 interaction in these contexts is confirmed by the use of PH-domain mutant forms of PLEKHA1/2 in these studies, and the mechanism of action of these proteins may be via the ability to restrain AKT activity, either via recruitment of proteins like the PTPL1 phosphatase, or via direct competition with AKT for available PtdIns(3,4)P_2 [1]. While current evidence suggests PLEKHA1/2 expression in platelets is likely to be low relative to lymphocytes [47, 50], even at limited global cellular abundance these high affinity PtdIns(3,4)P_2 effectors may be suitably poised to respond to the localised concentrations of PtdIns(3,4)P_2 described in cellular processes regulated by this phosphoinositide [3, 4, 18, 71], and their functional role in platelets warrants further investigation. It is worth noting that, while much published evidence supports the marked preferential affinity of PLEKHA1 and 2 for PtdIns(3,4)P_2 over other phosphoinositides [1, 2], it is clear that these proteins do demonstrate some affinity for PtdIns(3,4,5)P_3 [34, 55, 60, 72], but whether this is sufficient for functional responses to PtdIns(3,4,5)P_3 *in vivo* remains to be determined.

Beyond the aforementioned candidates, our interactome contained a broader set of PH domain-containing proteins, including RASA3, PHLDB1, MTMR5, GAB3, SWAP70, ADAP1 and PLEKHO2. With the exception of RASA3, the functional roles of these proteins in platelets remain unknown, yet it is noteworthy that, in other cell types, several have the ability to regulate the signalling and trafficking of integrins and other receptors, and to orchestrate dramatic changes in cytoskeletal organisation and membrane morphology. These processes are not only known to be

regulated by PtdIns(3,4)P_2 [1, 2, 14] and are critical for the function of a wealth of cell types, but are also of fundamental importance for platelet function in haemostasis and thrombosis, suggesting an important role for PtdIns(3,4)P_2 acting through such effectors in this cell type [73, 74]. This would correlate with alterations in membrane morphology, platelet contractility and thrombus organization observed with platelets lacking the PtdIns(3,4,5)P_3-phosphatase SHIP1, which demonstrate a loss of agonist-driven PtdIns(3,4)P_2 [32, 33, 64, 65].

PHLDB1 (also known as LL5α) has been shown previously to support AKT phosphorylation and GLUT4 translocation in adipocytes, to serve as a microtubule anchoring factor, and to support lamellipodial persistence and integrin internalisation in migrating tumour cells [42, 75, 76]. MTMR5 is an inactive phosphatase that has been proposed to act as a scaffolding protein and a GEF for RAB-family small GTPases [77-79], while ADAP1 is a GAP with dual PH domains; the first holds affinity for PtdIns(3,4,5)P_3, and the second for both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 [43]. ADAP1 has been shown to possess GAP activity for ARF6, a small GTPase with roles in vesicular trafficking (including platelet endocytosis [80]) and cytoskeletal arrangements, and accordingly ADAP1 has been implicated in control of the actin cytoskeleton, and the trafficking and surface expression of GPCRs and integrins [43, 81-84]. While GAB1 and 2 hold various functional roles in different cell types and are known to possess affinity for 3-phosphoinositides [85, 86], GAB3 has received more limited functional characterisation. It has been shown to regulate AKT activation and cancer cell proliferation in human glioma [87], and to our knowledge we provide the first evidence of the affinity of GAB3 for PtdIns(3,4)P_2. Although mice deficient in GAB3 display a normal platelet count [88], GAB3 appears to be well expressed in this cell type [47], and its functional role remains unknown. SWAP70 can serve as an actin-bundling protein, to regulate the actin cytoskeleton and lamellipodia formation in a PtdIns(3,4)P_2-dependent manner [44, 89, 90]. In contrast, PLEKHO2 is a poorly-characterised protein, so far known only to support macrophage survival [91], although it appears to be well expressed in platelets [47].

It is particularly interesting that our data suggest RASA3 to hold affinity for PtdIns(3,4)P_2. This RAS and RAP GAP is essential for platelet activation and lifespan, and serves as a key regulator of $\alpha_{IIb}\beta_3$ outside-in signalling downstream of PI3K [41, 48]. While RASA3 has demonstrated affinity for PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 [34, 41, 92] to our knowledge we provide the first evidence of its affinity for PtdIns(3,4)P_2, and this interaction may support PtdIns(3,4)P_2 in promoting sustained $\alpha_{IIb}\beta_3$ outside-in signalling. Indeed, given the association between integrin activation and PtdIns(3,4)P_2 generation in platelets, it is noteworthy that PtdIns(3,4)P_2 effectors such as RASA3, PHLDB1 [75, 76] and ADAP1 [84] identified in our screen are linked to integrin signalling and function.

In addition to PH domain-containing proteins, our dataset contains numerous other potentially novel PtdIns(3,4)P_2 effectors. If the majority of the proteins identified interact directly with PtdIns(3,4)P_2, as our network interaction analysis suggests, it is possible they do so via other protein domains, such as the PX domain of SNX2 [93], or via other clusters of basic amino acids within their sequence [51, 60]. Among these proteins is PNKD, which we previously revealed to possess affinity for PtdIns(3,4,5)P_3 [34]. PNKD can regulate the proliferation, adhesion and migration of cancer cells via a relationship with MLC2 and FAK (a key protein in platelet adhesive signalling), as well as the PtdIns(3,4,5)P_3/ PtdIns(3,4)P_2 effector AKT [94]. PNKD appears to be well expressed in platelets and may represent an important new 3-phosphoinositide effector regulating adhesive signalling in this cell type. Conversely, another protein we have shown to hold affinity for both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, ARMX3, can suppress non-small cell lung cancer invasion via suppression of AKT phosphorylation [95]. The identification of proteins such as SQOR, MICU1 and DECR1 suggests a potential role for PtdIns(3,4)P_2 in mitochondrial function, while the identification of receptors such as P2RX1, GPVI and F2RL3 suggests an importance for intramembrane lipid-protein interactions. Of note, P2RX1 has been shown previously to hold affinity for PtdIns(3,4)P_2 (in addition to PtdIns(4,5)P_2, known to regulate its function) [96], while GPVI and F2RL3 couple robustly to PI3K

signalling in platelets [34, 97]. The identification of F2RL3, in addition to GNAS2, is particularly interesting given recent work shedding light on the regulation of GPCR-G protein-coupling by $PtdInsP_2$ [98, 99].

5. Conclusions

It is tempting to envisage how many of the proteins identified in our screen can underpin both hitherto unknown, and recently-defined, roles for PtdIns(3,4)P_2 in a range of cell types, including the regulation of events like protein trafficking, membrane remodelling, and cell migration. Indeed it is intended that this study will support significant advancement of the understanding of PtdIns(3,4)P_2's cellular roles, by providing a springboard for the functional characterisation of the proteins identified and their relationship with this phosphoinositide. The value of this approach has been demonstrated previously for PtdIns(3,4)P_2 effectors such as PLEKHA1/2 and Lamellipodin, as well as for effectors for other phosphoinositides. Alongside effector characterisation, continued progress in the ability to visualise and quantify PtdIns(3,4)P_2 in distinct endogenous contexts, and to understand its synthesis and degradation, will be vital for a comprehensive understanding of this phospholipid. Determining the relationship between PtdIns(3,4)P_2's specific functional roles, and its significance as one component of a wider phosphoinositide network (e.g. as a breakdown product of PtdIns(3,4,5)P_3, or a precursor for PtdIns(3)P), will be interesting. It is clear these roles are not mutually exclusive, and much remains to be understood about PtdIns(3,4)P_2's functional interplay with PtdIns(3,4,5)P_3, since PtdIns(3,4)P_2 can both support and compete with PtdIns(3,4,5)P_3 for effectors, while each lipid can also act independently [16, 17]. A major experimental challenge is that PtdIns(3,4)P_2 function appears to be highly context-specific, potentially dependent on the cell type, the nature of the initiating stimulus, the presence of other positive or negative coincidence signals, and the available combination of effectors. The spatiotemporal properties of the PtdIns(3,4)P_2 signal will be critical in permitting it and other phosphoinositides to selectively regulate effectors, including individual AKT isoforms [18, 71], and it seems evident that PtdIns(3,4)P_2 localisation often dictates function [2-4]. A greater understanding of these factors, along with the potential functional significance of fatty acyl composition [11, 100, 101], will contribute to an improved understanding of phosphoinositide biology in health and disease.

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Authorship Contributions

T.N.D designed and performed experiments, analysed the data and wrote the manuscript. S.F.M designed and performed experiments, contributed to discussion and edited the manuscript. A.L.B. analysed data, contributed to discussion and edited the manuscript. E.W.A. designed and performed experiments. Y.J. performed experiments. M.C.W. and K.J.H. performed proteomics analysis and edited the manuscript. I.H. designed and performed experiments, contributed to discussion and co-wrote the manuscript.

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

Figure legends

Figure 1. Affinity proteomics workflow for the identification of human platelet PtdIns(3,4)P_2-binding proteins. *Platelets were isolated from the whole blood of healthy human donors, and lysed under mild non-denaturing conditions. Platelet lysates were incubated with PtdIns(3,4)P_2-coupled beads for the capture of PtdIns(3,4)P_2-binding proteins. For control samples, platelet lysates were either incubated with blank control beads, or preincubated with free PtdIns(3,4)P_2 prior to incubation with PtdIns(3,4)P_2-coupled beads, to assess binding specificity. For label-free proteomic analysis, captured proteins were eluted off the beads, gel-fractionated, and subjected to in-gel reduction, alkylation and trypsin-digestion prior to LC-MS/MS. For TMT analysis, proteins were subjected to on-bead reduction, alkylation and trypsin digestion, prior to labelling, pooling, high pH off-line RP-HPLC, and LC-MS3. Mass spectrometry data were subjected to quantitative filtering and assessed using a range of bioinformatics tools.*

Figure 2. Global analysis of the label-free proteomics data. *For initial visualisation and quality-control of the label-free proteomics data, T3PQ area values were log2 transformed, pooled, filtered, and imputed, as discussed in Supplementary Methods. Volcano plots were generated for PtdIns(3,4)P_2-coupled bead pull downs versus control blank bead pull downs (**A**), and for PtdIns(3,4)P_2-coupled bead pull downs versus PtdIns(3,4)P_2-coupled bead pull downs following preincubation of the platelet lysate with free PtdIns(3,4)P_2 (**B**), and presented displaying the difference in log2 means against negative log p values. A selection of well-characterised, and also potentially novel, PtdIns(3,4)P_2 effectors identified in this screen are highlighted. (**C**) Profile plots for selected proteins derived from the label-free analysis of a representative blood donor, displaying a range of protein behaviours against the background of all proteins for non-imputed data. PLEKHA1, DAPP1 and AKT2 represent well-established PtdIns(3,4)P_2 binding proteins. PLEKHA1 was captured specifically and abundantly on the PtdIns(3,4)P_2-coupled beads and thus passes data filtering following imputation of control values. DAPP1 displays some abundance in control samples, due to non-specific capture of this highly-abundant platelet protein, but displays significant enrichment on the PtdIns(3,4)P_2-coupled beads, requires no imputation, and passes data filtering. The archetypal PtdIns(3,4)P_2-binding protein AKT2 displays specific capture on the PtdIns(3,4)P_2-coupled beads, but is lost following imputation of control values due to its low abundance. UBAP2L is representative of many proteins displaying non-specific capture on the PtdIns(3,4)P_2-coupled beads versus blank beads, which were not susceptible to competition using free PtdIns(3,4)P_2. VDAC is representative of proteins captured non-specifically across all bead conditions. These observations contributed to the adoption of our final integrated strategy to define the PtdIns(3,4)P_2 interactome using both label-free and TMT analysis, as discussed in Supplementary Methods.*

Table 1. Selected components of the human platelet PtdIns(3,4)P_2 interactome. *For label-free data, presented are mean values for LC-MS/MS analysis of protein capture on PtdIns(3,4)P_2-coupled beads across three independent blood donors. Proteins are ranked based on mean abundance. For TMT data, presented are combined ratios from two independent blood donors for comparisons of PtdIns(3,4)P_2-coupled bead capture versus blank control bead capture (PIP2/Control), and PtdIns(3,4)P_2-coupled bead capture versus PtdIns(3,4)P_2-coupled bead capture following preincubation of the platelet lysate with free PtdIns(3,4)P_2 (PIP2/PIP2+). Shown is a spectrum of proteins identified in the screen, with the full data set presented in Table S1.*

Area, T3PQ abundance values; score, the total score of the protein, which is the sum of all peptide XCorr values for that protein above the specified score threshold; coverage, the percentage of the protein sequence covered by the identified peptides; peptides, the number of distinct peptide sequences identified for the protein; PSM, the total number of identified peptide sequences for the protein, including those redundantly identified.

Figure 3. Immunoblotting of selected PtdIns(3,4)P_2 effectors captured on PtdIns(3,4)P_2-coupled beads. *Affinity capture of PtdIns(3,4)P_2-binding proteins was carried out as detailed for label-free proteomics experiments, and eluted proteins were separated by SDS-PAGE and subjected to LI-COR immunoblotting using specific antibodies. Input represents 3% of affinity capture input. GAPDH represents a negative control. Results are representative of three independent experiments.*

Figure 4. PH domain analysis of identified PtdIns(3,4)P_2-binding proteins. *(**A**) Amino acid sequence alignment of the PH domains of proteins identified in the PtdIns(3,4)P_2 interactome confirms the presence of conserved lysine (K) and arginine (R) residues (red boxes), including key components of the putative PtdIns(3,4)P_2/PtdIns(3,4,5)P_3-binding pocket in the β1-β2 strand region. Basic residues at other positions are also likely to contribute to PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 binding. The key lysine and arginine residues that contribute to SWAP70 and OSBL8's non-canonical mode of binding to PtdIns(3,4)P_2/ PtdIns(3,4,5)P_3 are indicated in blue boxes. Additional glycine (G), phenylalanine (F), tyrosine (Y) and tryptophan (W) residues supporting the alignment are indicated (orange boxes), although these are not necessarily relevant for 3-phosphoinositide binding. For PLEKHA1/2 and ADAP1, the C-PH domains are presented, which have been previously demonstrated to mediate the PtdIns(3,4)P_2-binding of these proteins. (**B**) Domain analysis of the PtdIns(3,4)P_2 interactome reveals the significant enrichment of pleckstrin homology (PH) domain-containing proteins. The number of PH domain-containing proteins in the interactome, the fold enrichment, and a Benjamini p value are shown.*

Figure 5. ***Characterisation of the MTMR5 PH domain*** *(**A**) Assessment of the structure of the PH domain of mouse MTMR5 (PDB 1V5U) reveals a characteristic seven-stranded β-sandwich with a C-terminal α-helix. Mapping of the most highly conserved basic residues (red) from the PH domain alignment in Figure 4 onto this structure (these residues are identical between the human and mouse MTMR5 PH domain) suggests a phosphoinositide-binding pocket comparable to that previously defined for other PtdIns(3,4)P_2/PtdIns(3,4,5)P_3 effectors. (B) Coommassie-stained SDS-PAGE gel of the purified human MTMR5 PH domain expressed as a GST-fusion protein. (C) The GST-tagged MTMR5 PH domain shown in (B) was assessed for its phosphoinositide selectivity using a phosphoinositide binding array, confirming affinity for PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Of note, MTMR5 was identified with one blood donor in our previous screen for PtdIns(3,4,5)P_3 effectors [34], but was not identified with the other donors, potentially due to low affinity for this phosphoinositide. Results are representative of three independent experiments.*

Figure 6. Interaction network analysis of the PtdIns(3,4)P_2 interactome. *To identify interactions within the experimentally-derived PtdIns(3,4)P_2 interactome and hence to determine whether some proteins identified may not interact directly with PtdIns(3,4)P_2 , the complete protein set was subjected to analysis using STRING version 11, screening for experimentally-determined (pink connections) and database-curated (blue connections) interactions, with a confidence score of 0.9.*

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Table 1. Selected components of the human platelet PtdIns(3,4)P_2 interactome. *For label-free data, presented are mean values for LC-MS/MS analysis of protein capture on PtdIns(3,4)P_2-coupled beads across three independent blood donors. Proteins are ranked based on mean abundance. For TMT data, presented are combined ratios from two independent blood donors for comparisons of PtdIns(3,4)P_2-coupled bead capture versus blank control bead capture (PIP2/Control), and PtdIns(3,4)P_2-coupled bead capture versus PtdIns(3,4)P_2-coupled bead capture following preincubation of the platelet lysate with free PtdIns(3,4)P_2 (PIP2/PIP2+). Shown is a spectrum of proteins identified in the screen, with the full data set presented in Table S1.*

Area, T3PQ abundance values; score, the total score of the protein, which is the sum of all peptide XCorr values for that protein above the specified score threshold; coverage, the percentage of the protein sequence covered by the identified peptides; peptides, the number of distinct peptide sequences identified for the protein; PSM, the total number of identified peptide sequences for the protein, including those redundantly identified.

			Label-free					TMT	
Accession	Gene	Description	Mean area	Mean score	Mean coverage (%)	Mean peptides	Mean PSM	PIP2/Control	PIP2/PIP2+
Q9UN19	DAPP1	Dual adapter for phosphotyrosine and 3-phosphoinositide / BAM32	6.17E+09	557.1	77.0	22.3	188.3	52.8	18.7
Q8N490	PNKD	Probable hydrolase PNKD	1.49E+08	53.6	32.8	10.0	18.3	7.3	4.5
Q14644	RASA3	Ras GTPase-activating protein 3	1.34E+08	129.3	46.6	31.7	45.7	35.9	5.6
Q9HB19	PLEKHA2	Pleckstrin homology domain-containing family A member 2 / TAPP2	1.07E+08	67.8	47.0	14.7	25.0	82.4	11.2
Q9HB21	PLEKHA1	Pleckstrin homology domain-containing family A member 1 / TAPP1	5.85E+07	69.4	48.0	15.3	28.0	99.6	16.3
Q86UU1	PHLDB1	Pleckstrin homology-like domain family B member 1 / LL5α	3.91E+07	38.1	11.2	11.7	12.3	9.7	4.0
Q9UH62	ARMCX3	Armadillo repeat-containing X-linked protein 3	2.69E+07	34.8	34.4	10.3	12.3	5.8	3.2
O95248	SBF1	Myotubularin-related protein 5 / MTMR5	2.32E+07	72.6	17.5	21.7	24.7	10.6	2.1
Q53H12	AGK	Acylglycerol kinase	2.10E+07	27.4	33.3	9.7	10.3	6.3	8.3
Q13613	MTMR1	Myotubularin-related protein 1	2.02E+07	31.0	22.4	11.0	13.0	10.6	2.3
Q9Y6N5	SQOR	Sulfide:quinone oxidoreductase	1.76E+07	21.3	19.7	7.3	8.0	4.9	5.2
P53007	SLC25A1	Tricarboxylate transport protein / TXTP	1.52E+07	7.9	13.8	4.0	4.3	7.2	3.9
Q16698	DECR1	2,4-dienoyl-CoA reductase	1.18E+07	13.3	18.2	4.7	4.7	5.3	4.4
Q8WWW8	GAB3	GRB2-associated-binding protein 3	8.53E+06	12.6	8.2	4.7	5.3	7.9	4.0
Q9UH65	SWAP70	Switch-associated protein 70	3.92E+06	5.3	3.8	2.3	2.3	5.0	1.5
O75689	ADAP1	Arf-GAP with dual PH domain-containing protein 1	3.43E+06	6.0	8.9	3.3	3.3	34.5	11.5
Q8TD55	PLEKHO2	Pleckstrin homology domain-containing family O member 2	2.32E+06	9.5	9.0	2.3	3.0	4.0	1.7

		/ PKHO2							
Q9BZ67	FRMD8	FERM domain-containing protein 8	1.81E+06	2.4	3.8	1.7	1.7	4.9	2.8
Q9BZF1	OSBPL8	Oxysterol-binding protein-related protein 8 / OSBL8	9.81E+05	2.4	1.2	1.0	1.0	6.2	1.6
P31751	AKT2	RAC-beta serine/threonine-protein kinase / PKBβ	6.23E+05	4.5	4.6	2.3	2.3	9.6	8.9

Highlights

- A global screen for PtdIns(3,4)P_2-interacting proteins in human platelets.
- PtdIns(3,4)P_2 interactors span a range of functional classes, including regulators of protein trafficking and cytoskeletal organisation.
- The PH domain of MTMR5 shows affinity for PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3.

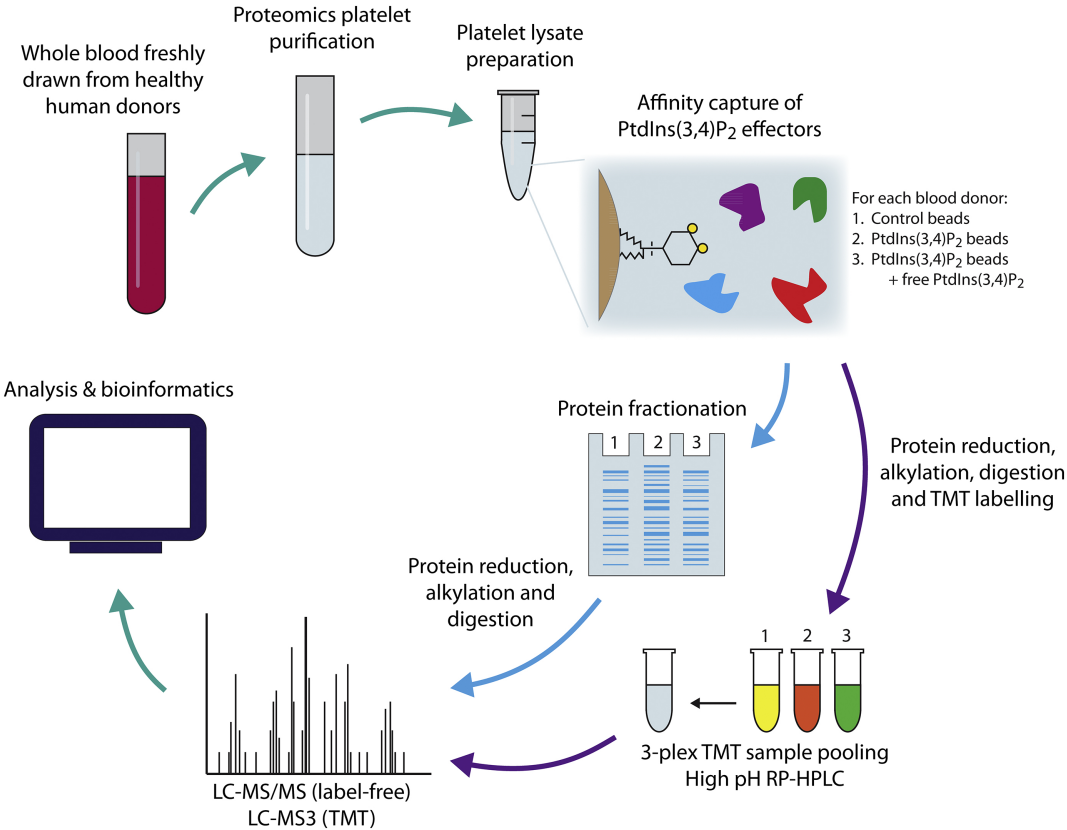

Whole blood freshly drawn from healthy human donors
Proteomics platelet purification
Platelet lysate preparation
Affinity capture of PtdIns(3,4)P2 effectors
For each blood donor:
1. Control beads
2. PtdIns(3,4)P2 beads
3. PtdIns(3,4)P2 beads + free PtdIns(3,4)P2
Analysis & bioinformatics
Protein fractionation
1
2
3
Protein reduction, alkylation, digestion and TMT labelling
Protein reduction, alkylation and digestion
1
2
3
3-plex TMT sample pooling
High pH RP-HPLC
LC-MS/MS (label-free)
LC-MS3 (TMT)

Figure 1

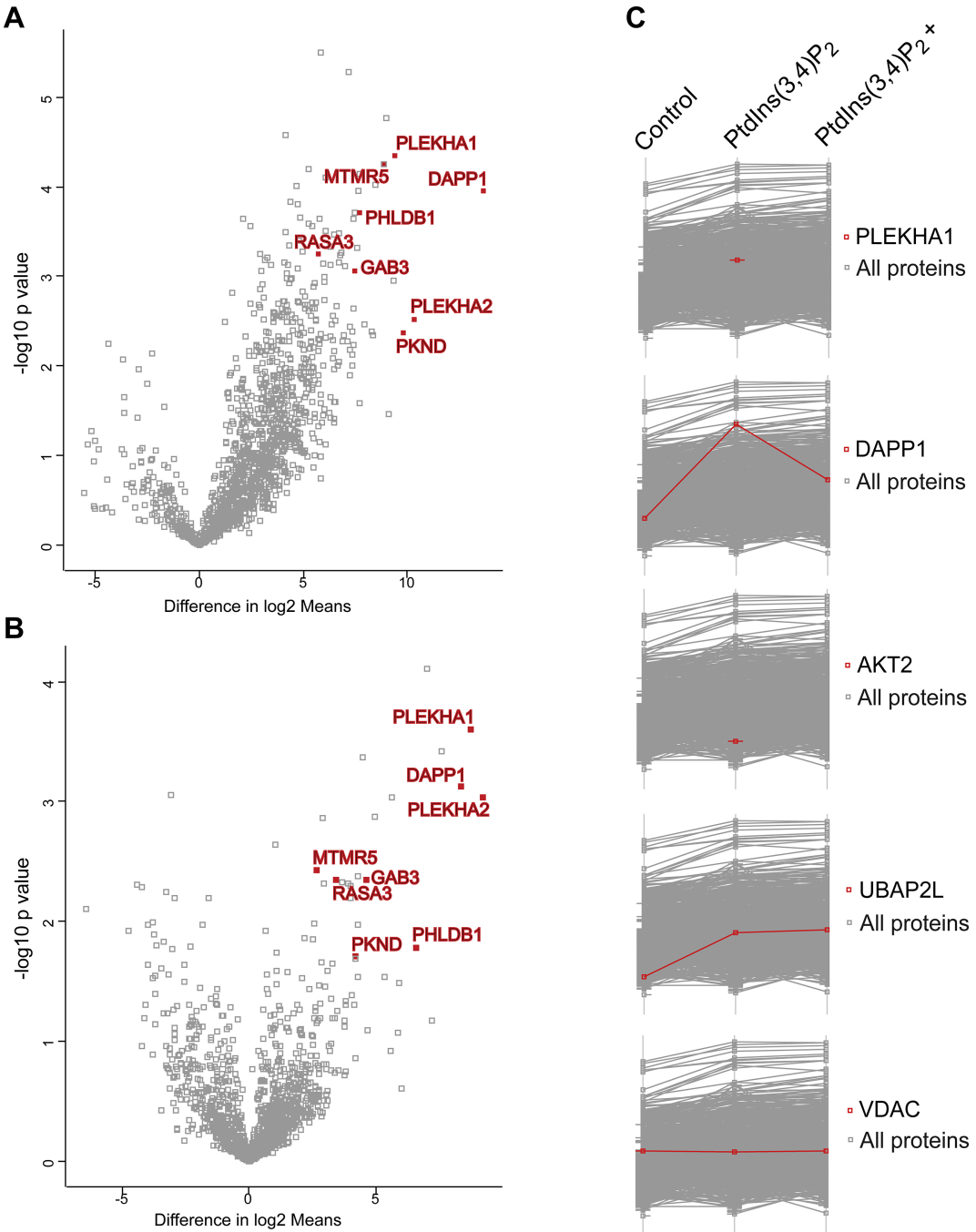

A
-log10 p value
Difference in log2 Means
PLEKHA1
MTMR5
DAPP1
PHLDB1
RASA3
GAB3
PLEKHA2
PKND
B
-log10 p value
Difference in log2 Means
PLEKHA1
DAPP1
PLEKHA2
MTMR5
GAB3
RASA3
PKND
PHLDB1
C
Control
PtdIns(3,4)P2
PtdIns(3,4)P2+
PLEKHA1
All proteins
DAPP1
All proteins
AKT2
All proteins
UBAP2L
All proteins
VDAC
All proteins

Figure 2

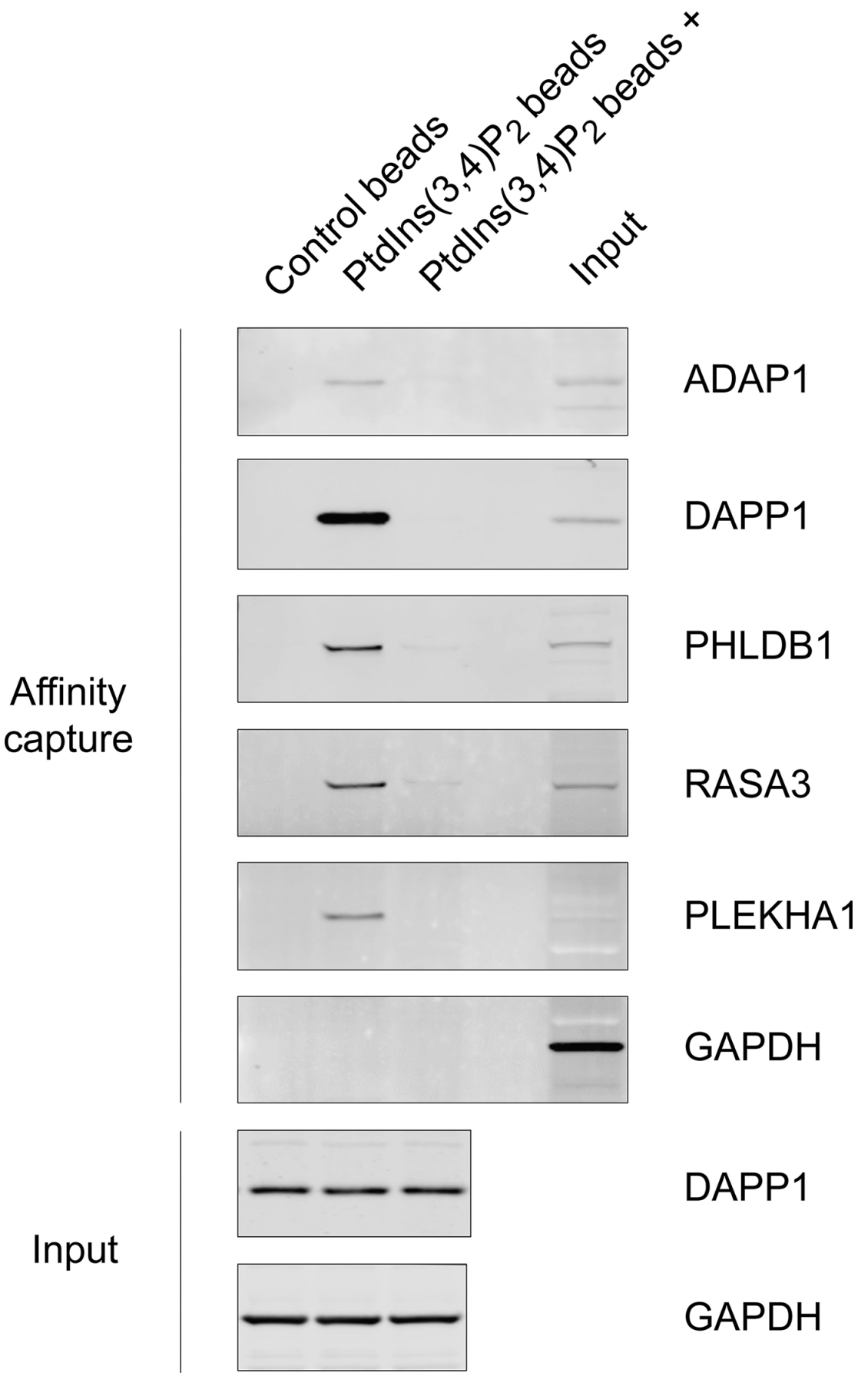
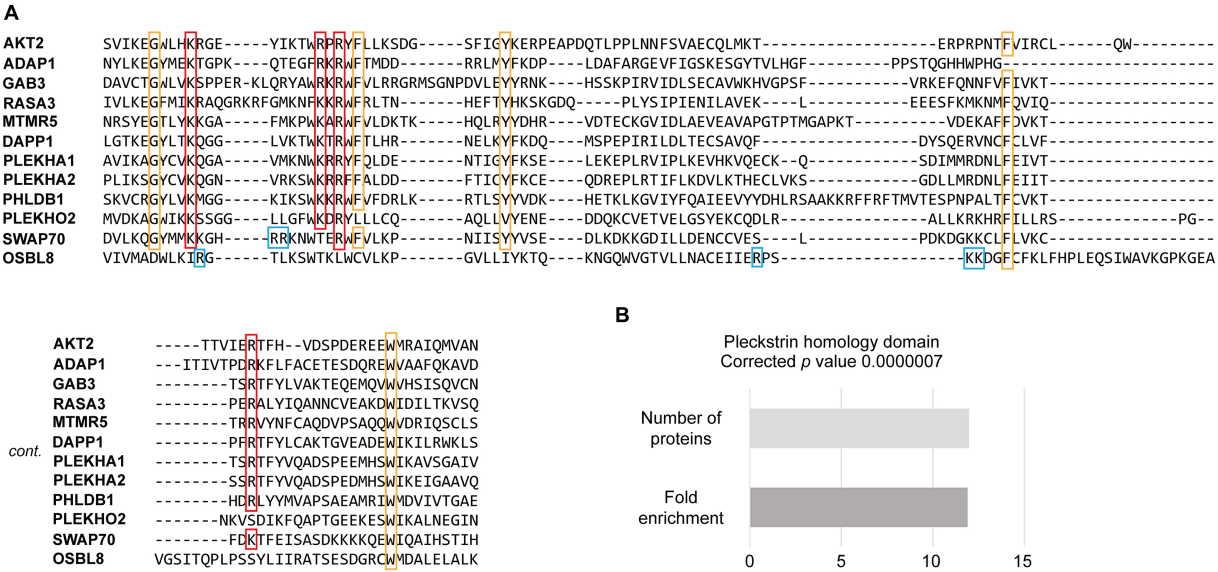

Control beads
PtdIns(3,4)P2 beads
PtdIns(3,4)P2 beads +
Input
ADAP1
DAPP1
PHLDB1
RASA3
PLEKHA1
GAPDH
Affinity capture
DAPP1
GAPDH
Input

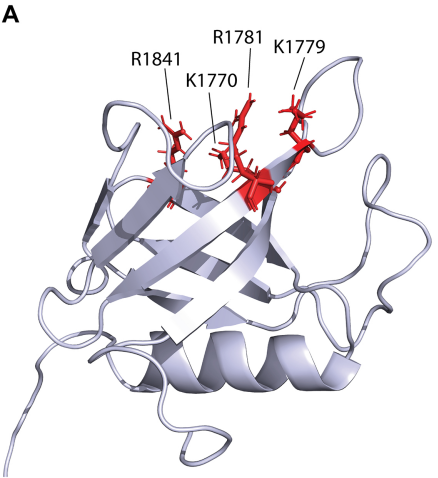
Figure 3

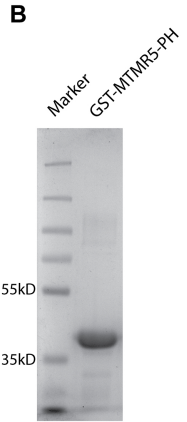


cont.

```
AKT2  -----TTVIERTFH--VDSPDEREEWMRAIQMVAN
ADAP1 ---ITIVTPDRKFLFACETESDQREWVAAFQKAVD
GAB3  --------TSRTFYLVAKTEQEMQVWVHSISQVCN
RASA3 --------PERALYIQANNCVEAKDWIDILTKVSQ
MTMR5 --------TRRVYNFCAQDVPSAQQWVDRIQSCLS
DAPP1 --------PFRTFYLCAKTGVEADEWIKILRWKLS
PLEKHA1 --------TSRTFYVQADSPEEMHSWIKAVSGAIV
PLEKHA2 --------SSRTFYVQADSPEDMHSWIKEIGAAVQ
PHLDB1 --------HDRLYYMVAPSAEAMRIWMDVIVTGAE
PLEKHO2 -------NKVSDIKFQAPTGEEKESWIKALNEGIN
SWAP70 --------FDKTFEISASDKKKKQEWIQAIHSTIH
OSBL8 VGSITQPLPSSYLIIRATSESDGRCWMDALELALK
```

Figure 4


A
R1841
K1770
R1781
K1779


B
Marker
GST-MTMR5-PH
55kD
35kD

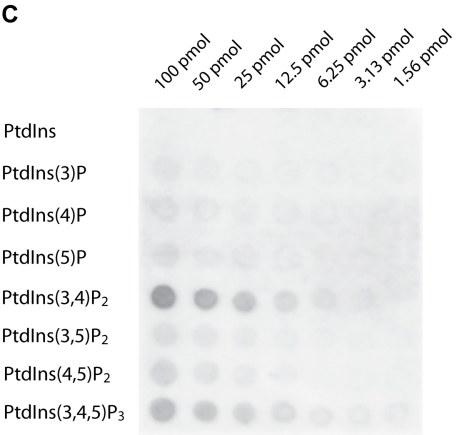
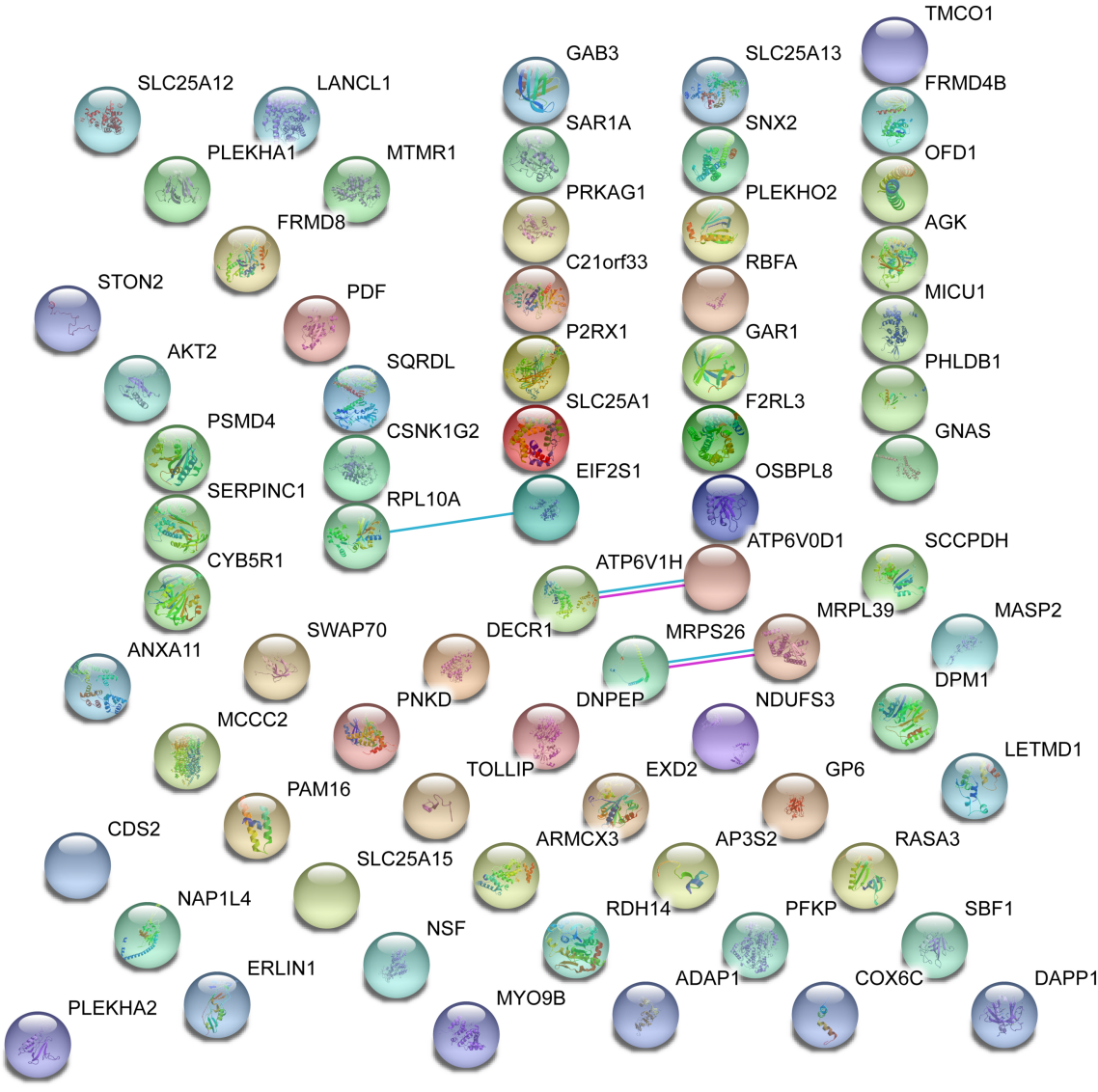

C
100 pmol
50 pmol
25 pmol
12.5 pmol
6.25 pmol
3.13 pmol
1.56 pmol
PtdIns
PtdIns(3)P
PtdIns(4)P
PtdIns(5)P
PtdIns(3,4)P2
PtdIns(3,5)P2
PtdIns(4,5)P2
PtdIns(3,4,5)P3

Figure 5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PLEKHA1
MTMR1
FRMD8
STON2
PDF
AKT2
SQRDL
PSMD4
CSNK1G2
SERPINC1
RPL10A
CYB5R1
ANXA11
SWAP70
MCCC2
PAM16
CDS2
NAP1L4
ERLIN1
PLEKHA2
GAB3
SAR1A
PRKAG1
C21orf33
P2RX1
SLC25A1
EIF2S1
DECR1
PNKD
TOLLIP
ARMCX3
SLC25A15
NSF
MYO9B
SLC25A13
SNX2
PLEKHO2
RBFA
GAR1
F2RL3
OSBPL8
ATP6V0D1
ATP6V1H
MRPS26
DNPEP
NDUFS3
EXD2
AP3S2
RDH14
ADAP1
TMCO1
FRMD4B
OFD1
AGK
MICU1
PHLDB1
GNAS
SCCPDH
MRPL39
MASP2
DPM1
LETMD1
GP6
RASA3
PFKP
SBF1
COX6C
DAPP1

Figure 6